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# *In vitro* Susceptibility of *Leishmania donovani* to Miltefosine in Indian Visceral Leishmaniasis

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*Abstract.* Promastigote miltefosine (MIL) susceptibility was performed on *Leishmania donovani* isolates from Indian patients with visceral leishmaniasis treated with MIL. Isolates that were obtained before the onset of MIL treatment, after completion of treatment (29th day), or at the time of treatment failure, were screened using *in vitro* promastigote assay. The MIL susceptibility of the pre-treatment isolates ($N = 24$, mean $IC_{50} \pm$ SEM = $3.74 \pm 0.38$ μM) was significantly higher than that of the post-treatment group ($N = 26$, mean $IC_{50} \pm$ SEM = $6.15 \pm 0.52$ μM; $P = 0.0006$) but was similar in the cured patients ($N = 22$, mean $IC_{50} \pm$ SEM = $5.58 \pm 0.56$ μM) and those who failed treatment ($N = 28$, mean $IC_{50} \pm$ SEM = $4.53 \pm 0.47$ μM). The pre/post-treatment results thus showed a 2-fold difference, whereas isolated from cured versus failed patients showed a similar susceptibility, suggesting that this higher tolerance is not responsible for MIL-treatment failure. Our work highlights the need for careful monitoring of MIL susceptibility for implementation in national VL elimination programs.

## INTRODUCTION

Visceral leishmaniasis (VL or kala-azar) affects 79 countries, with an estimated 58,000 new cases and 10% deaths occurring worldwide.[1,2] More than 90% of VL cases occur in India, Bangladesh, South Sudan, Sudan, Ethiopia, and Brazil, and 50% of the global VL is concentrated in the north-eastern Indian state of Bihar.[1] Resistance to antimonials, severe side effects, parenteral administration, and high costs of anti-leishmanial drugs are major issues in the treatment of VL. Early treatment is the key to the Kala-Azar Elimination Program (KAEP) in the Indian subcontinent, launched by the governments of India, Nepal, and Bangladesh to reduce the annual VL incidence to < 1 per 10,000 inhabitants by the year 2015.[1,3,4] Miltefosine (MIL), paromomycin, and amphotericin B are the only drugs that are currently effective in the Indian subcontinent, especially in Bihar.[5] Of these, MIL was chosen for the KAEP because of its oral use and subsequent easier implementation in the region. Clinical trials in India, undertaken during 1997–2000, showed a cure rate of 94% among VL patients treated with oral MIL for 4 weeks.[6,7] However, recent studies found significant declines in the cure rate of MIL caused by an increased relapse rate.[8,9]

Anthroponotic transmission of *Leishmania donovani* as well as the long half-life of MIL and the prolonged treatment duration constitute a serious risk toward the emergence and spread of MIL-resistance. In a recent study, isolates from relapsed VL and PKDL (post kala-azar dermal leishmaniasis) cases were shown to be more tolerant to MIL (but not yet resistant) than the pre-treatment isolates, thereby emphasizing the need for close monitoring of cases under MIL treatment. In that study, drug susceptibility testing was done with intracellular amastigotes, which is complex and time-consuming. However, recently it was shown that the *in vitro* susceptibility of promastigotes could be correlated to the *in vitro* susceptibility of amastigotes, allowing the development and validation of a fluorometric resazurin assay using promastigotes to determine parasite MIL susceptibility.[10–12] This simple biological tool is less laborious than the *in vitro* amastigote assay and is therefore more fit for high-throughput screening of MIL susceptibility of clinical isolates. In this study, this promastigote assay was used in the context of a clinical study on the efficacy of MIL in Bihar to assess the impact of parasite susceptibility to MIL (before and after treatment) on MIL-treatment outcome.

## MATERIAL AND METHODS

**Ethics statement.** The study was approved by the Ethics committees of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, and the Institute of Tropical Medicine, Antwerp, Belgium. Written informed consent was obtained from all the subjects enrolled into the study.

**Patient recruitment and treatment.** From September 2009 to November 2010, we recruited 567 patients with VL in this open-label study at Kala Azar Medical Research Center (KAMRC) Muzaffarpur, field site of Banaras Hindu University. Patients between the ages of 6 and 70 years qualified for the enrollment of MIL treatment, if they had symptoms and signs of kala-azar (fever, splenomegaly, and weight loss) and presence of amastigotes in the splenic smears by microscopy. Every patient received MIL, 50 mg capsule twice a day for adults weighing ≥ 25 kg, 50 mg once daily for those weighing < 25 kg, and 2.5 mg/kg/day for children < 12 years of age for 28 days, in directly observed conditions. Patients were followed up for 6 months for determination of final cure, and at 1 year for any late relapses. Those relapsing after an initial cure were given rescue treatment with amphotericin B. "Initial cure" was defined as resolution of fever, regression of splenic enlargement, and recovery of laboratory parameters toward normal at the end of treatment and absence or grade 1 presence of parasites in splenic smears taken after the last day of treatment. Those with grade 1 presence of parasites were re-evaluated 2 weeks later[8] and if there were grade 0 (no) parasites at that time, these subjects were considered as initial cure, and were not administered rescue therapy. Thus, post-treatment parasites could be obtained from patients who eventually show complete cure.

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**Culture media and MIL.** Adenosine, folic acid, biotin, hemin, $NaHCO_3$ for medium, and resazurin dye were purchased from Sigma, whereas M199, RPMI-1640 medium, L-glutamine, and fetal bovine serum (FBS) were supplied by Invitrogen. Preservative-free crystalline form of MIL (Batch: 1149149 obtained from Paladin) was used for susceptibility experiment.

**Isolation and culture of *L. donovani* promastigotes.** For obtaining clinical isolates of *L. donovani,* splenic material from the patients was inoculated in M199 medium with 10% heat-inactivated FBS in NNN (Novy-MacNeal-Nicolle) tube and incubated at 25°C in a BOD incubator.[13] Antibiotics (Penicillin-50 U/mL and streptomycin-50 μg/mL; Gibco) were used at isolation time and during the maintenance of clinical isolates. Promastigotes were propagated for secondary culture in M199 after 7 days of incubation in single phase liquid medium. These secondary/tertiary cultures of promastigotes were used for MIL susceptibility screening and subsequently remaining parasites were cryopreserved. All clinical isolates were confirmed to be *L. donovani* by typing with Hsp70 polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[14]; we selected two types of clinical isolates for the MIL susceptibility experiments on the basis of the past and present clinical

TABLE 1

Miltefosine susceptibility of clinical *Leishmania donovani* isolates from Indian miltefosine treated visceral leishmaniasis patients

| Isolate code | Age | Sex | MIL IC50 (μM) [95% CI] | Treatment response to MIL | Isolate pre- or post-MIL treatment | Number of months since initiation last MIL-treatment that isolate was obtained |
|---|---|---|---|---|---|---|
| MHOM/IN/10/BHU783/0 | 40 | M | 3.30 [2.87–3.79] | cure | pre | N/A |
| MHOM/IN/09/BHU774/0 | 62 | M | 7.62 [6.98–8.31] | fail | pre | N/A |
| MHOM/IN/10/BHU790/0 | 12 | F | 3.52 [3.1–3.99] | fail | pre | N/A |
| MHOM/IN/09/BHU796/1 | 12 | F | 3.23 [2.6–4.03] | cure | post | 1 months |
| MHOM/IN/09/BHU797/0 | 24 | M | 3.52 [3.1–3.99] | cure | pre | N/A |
| MHOM/IN/10/BHU798/0 | 48 | M | 2.75 [2.53–2.98] | cure | pre | N/A |
| MHOM/IN/10/BHU800/0 | 35 | M | 3.24 [3.03–3.45] | cure | pre | N/A |
| **MHOM/IN/10/BHU807/0** | 12 | F | 3.36 [3.1–3.63] | cure | pre | N/A |
| **MHOM/IN/10/BHU807/1** | 12 | F | 5.34 [5.08–5.62] | cure | post | 1 month |
| MHOM/IN/10/BHU808/0 | 12 | M | 3.11 [3–3.24] | cure | pre | N/A |
| MHOM/IN/10/BHU812/0 | 35 | F | 3.17 [2.13–4.73] | cure | pre | N/A |
| MHOM/IN/10/BHU814/1 | 12 | F | 7.92 [7.67–8.18] | cure | post | N/A |
| **MHOM/IN/10/BHU815/0** | 12 | F | 6.41 [6.06–6.79] | cure | pre | N/A |
| **MHOM/IN/10/BHU815/1** | 12 | F | 6.90 [6.55–7.27] | cure | post | 1 month |
| MHOM/IN/10/BHU816/1 | 12 | F | 2.51 [2.2–2.88] | cure | post | 1 months |
| MHOM/IN/10/BHU824/0 | 14 | M | 2.47 [2.24–2.71] | fail | pre | N/A |
| **MHOM/IN/10/BHU828/0** | 10 | M | 2.76 [2.62–2.9] | fail | pre | N/A |
| **MHOM/IN/10/BHU828/7** | 11 | M | 6.45 [6.13–6.8] | fail | post | 7 months |
| MHOM/IN/10/BHU830/0 | 35 | M | 4.21 [3.95–4.49] | fail | pre | N/A |
| MHOM/IN/10/BHU844/0 | 7 | F | 2.70 [2.36–3.09] | fail | pre | N/A |
| MHOM/IN/10/BHU848/0 | 35 | F | 1.97 [1.71–2.26] | fail | pre | N/A |
| MHOM/IN/10/BHU860/0 | 7 | M | 2.03 [1.8–2.29] | fail | pre | N/A |
| MHOM/IN/10/BHU861/0 | 8 | M | 1.80 [1.59–2.04] | fail | pre | N/A |
| **MHOM/IN/10/BHU869/0** | 10 | F | 6.35 [5.84–6.89] | cure | pre | N/A |
| **MHOM/IN/10/BHU869/1** | 10 | F | 10.88 [9.99–11.83] | cure | post | 1 month |
| MHOM/IN/10/BHU872/6 | 9 | M | 7.05 [5.04–9.86] | fail | post | 6 months |
| MHOM/IN/10/BHU902/0 | 7 | M | 4.19 [3.71–4.73] | fail | post | 8 months |
| MHOM/IN/10/BHU931/0 | 6 | M | 3.19 [2.83–3.6] | fail & cure* | pre/post* | 4 months |
| MHOM/IN/10/BHU965/0 | 12 | M | 2.26 [2.13–2.4] | fail | pre | N/A |
| MHOM/IN/10/BHU975/0 | 12 | M | 1.37 [1.2–1.56] | fail | pre | N/A |
| MHOM/IN/10/BHU982/0 | 10 | M | 8.23 [7.39–9.17] | fail | pre | N/A |
| **MHOM/IN/10/BHU994/0** | 60 | M | 4.69 [4.09–5.38] | cure | pre | N/A |
| **MHOM/IN/10/BHU994/1** | 60 | M | 4.99 [4.07–6.13] | cure | post | 1 month |
| MHOM/IN/10/BHU1052/0 | 65 | M | 3.50 [3.31–3.69] | fail & cure* | pre/post* | 2 months |
| MHOM/IN/10/BHU1087/0 | 56 | M | 4.53 [4.07–5.05] | fail | post | 4 months |
| MHOM/IN/10/BHU1011/0 | 9 | M | 5.86 [5.08–6.75] | fail | pre | N/A |
| MHOM/IN/10/BHU1032/0 | 34 | M | 3.03 [2.77–3.31] | fail | pre | N/A |
| MHOM/IN/10/BHU1042/1 | 6 | M | 11.09 [9.77–12.58] | cure | post | 1 months |
| MHOM/IN/10/BHU1062/4 | 7 | F | 12.11 [11.39–12.88] | fail | post | 4 months |
| MHOM/IN/10/BHU1064/0 | 9 | F | 4.19 [3.71–4.73] | fail | post | 4 months |
| MHOM/IN/10/BHU1065/0 | 20 | M | 7.62 [6.98–8.31] | fail | post | 2 months |
| **MHOM/IN/10/BHU1080/1** | 12 | M | 9.30 [8.85–9.76] | cure | post | 1 month |
| **MHOM/IN/10/BHU1080/3** | 12 | M | 4.50 [4.21–4.83] | fail | post | 3 months |
| MHOM/IN/10/BHU1092/0 | 32 | M | 2.88 [2.57–3.23] | fail | post | 4 months |
| MHOM/IN/10/BHU1104/0 | 12 | M | 2.01 [1.9–2.13] | fail | post | 9 months |
| MHOM/IN/10/BHU1113/7 | 35 | M | 3.53 [2.8–4.45] | fail | post | 7 months |
| MHOM/IN/10/BHU1142/6 | 6 | M | 5.34 [5.15–5.54] | fail | post | 6 months |
| MHOM/IN/10/BHU1146/0 | 13 | F | 4.46 [3.7–5.38] | fail & cure* | pre/post* | 8 months |
| MHOM/IN/10/BHU1152/1 | 35 | F | 7.10 [5.61–8.99] | cure | post | 1 months |
| MHOM/IN/10/BHU1153/1 | 11 | M | 7.61 [5.77–10.05] | cure | post | 1 months |
| MHOM/IN/10/BHU1154/1 | 50 | M | 5.94 [4.5–7.84] | cure | post | 1 months |
| MHOM/IN/10/BHU1161/0 | 9 | F | 5.49 [4.54–6.65] | fail | post | 3 months |
| MHOM/IN/10/BHU1163/4 | 7 | M | 7.11 [5.96–8.47] | fail | post | 4 months |

*These isolates were grown from post-treatment splenic aspirates. They did not receive rescue treatment because they achieved initial cure.
Bold-paired isolates obtained from the same patient at the onset of treatment and at the time of relapse.
N/A = not applicable.

response of the corresponding VL patients. We isolated clinical isolates from VL patients at different timescales of MIL drug trial. First, isolates obtained before the onset of treatment (labeled BHUXXX/0 in Table 1). Second, isolates obtained at the completion of the treatment when splenic aspiration was repeated for the test of cure (further called post-treatment isolates and labeled BHUXXX/1). Table 1 summarizes all isolate-specific data such as the type of sample and treatment outcome of the patient from whom the isolate was obtained. Third, isolates from patients who failed after initial cure (further called treatment failure isolates and labeled BHUXXX/n, n standing for the number of months when treatment failed occurred).

**MIL susceptibility assay for *L. donovani* isolates.** The main stock of MIL (10 mg/mL) was prepared in 0.22 micrometer filter sterilized 1X phosphate buffered saline (PBS), and was stored in 1 mL aliquots in –20°C for up to 3 months. Promastigotes of *L. donovani* ($1 \times 10^5$) were grown on flat bottom 96-well plates for the estimation of susceptibility to MIL. Log-phase promastigotes were harvested and seeded into the wells in 200 μL M199 medium. The upper four rows were used for one strain and the lower four rows were used for another strain. Simultaneously, MIL was added to the test wells in quadruplicate for 11 different concentrations (ranging from 0.38 to 392.57 μM in 1:2 serial dilutions) and incubated for 72 hours at 25°C. Positive (with parasites) and negative (without parasites) control wells were not exposed to MIL. After the completion of incubation time, 50 μL of the resazurin dye was added to each well (50 μg/mL) and incubated for 24 hours. After this incubation, the fluorescence was measured using the filter combination 550–590 nm.

**Calculation of $IC_{50}$ and statistical analysis.** The relative amount of viable parasites in the drug-treated and the control wells were estimated by the resazurin assay for each of the quadruplicate wells for different concentrations. Taking the mean of the control wells to equate to 100% survival, all the different concentrations were converted into percentages. GraphPad Prism5 was used for $IC_{50}$ calculation, using a sigmoidal dose-response model with variable slope, and statistical analysis. An unpaired *t* test (two-tailed) was applied to determine the significance between groups of isolates.

## RESULTS

The MIL susceptibility was screened at the promastigote stage for 53 clinical *L. donovani* isolates, which included 24 pre-treatment, 26 post-treatment, and 3 isolated in between 2 MIL-treatment regimens (described as pre/post in Table 1). Of these, 28 were isolated from patients showing MIL-fail, 22 from MIL-cures, and 3 from patients that showed an earlier MIL-fail, but were later definitively cured by a later round of MIL treatment. The mean $IC_{50} \pm$ SEM of 28 isolates from MIL-treatment failure was 4.53 ± 0.47 μM, which was similar to the mean $IC_{50} \pm$ SEM of 22 isolates from MIL-cure subjects (5.58 ± 0.56, $P = 0.1564$). Furthermore, pre-treatment isolates from patients destined to cure ($N = 10$) showed a similar $IC_{50} \pm$ SEM when compared with pre-treatment isolates of patients destined to fail ($N = 14$) (3.99 ± 0.43 versus 3.56 ± 0.58, $P = 0.5859$). Post-treatment isolates from patients destined to cure ($N = 12$) also showed a similar $IC_{50} \pm$ SEM when compared with post-treatment isolates of patients destined to fail MIL treatment ($N = 14$) from (6.90 ± 0.78 versus 5.50 ± 0.67, $P = 0.1844$). Interestingly, all pre-treatment isolates ($N = 24$) showed a mean $IC_{50} \pm$ SEM of 3.74 ± 0.38 μM, whereas all post-treatment isolates ($N = 26$) showed a significantly ($P = 0.0006$) higher mean $IC_{50} \pm$ SEM of 6.15 ± 0.52 μM. A similar observation was made when the pre- and post-treatment isolates where compared for each treatment outcome group separately. Table 1 and Figure 1 summarize all characteristics of all tested isolates.

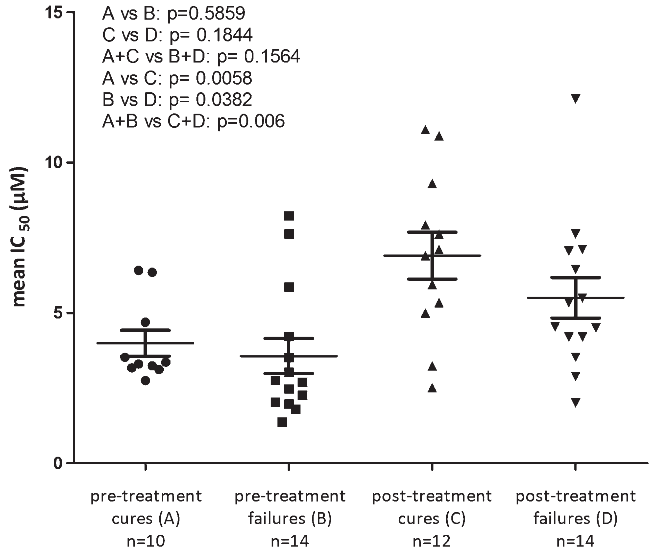


FIGURE 1. Miltefosine susceptibility assay was performed between the group of *Leishmania donovani* isolates patients destined to cure and patients destined to fail from pre- and post-treatment group of subjects.

## DISCUSSION

Treatment outcome in VL is a complex phenomenon that depends essentially on the interaction among the drug, the parasite, and the host. The standard method to track parasite susceptibility to a drug is to perform *in vitro* susceptibility assays on the amastigote stage of *Leishmania*. In the case of MIL however, *in vitro* promastigote susceptibility has proven to correlate with *in vitro* amastigote susceptibility[12]; in this study we applied this previously validated *in vitro* promastigote assay to assess the MIL susceptibility of parasites isolated from patients that responded differently to MIL treatment. One of the strengths of this study is that some isolates could be obtained at the end of treatment of patients that were clinically cured, which ensures a more realistic representation of post-treatment samples compared with studies that only have post-treatment samples taken at the time of clinical relapse. Using the sample set described in Table 1, we observed that *L. donovani* isolates obtained post-treatment from VL patients showed a significantly higher tolerance toward MIL compared with pre-treatment isolates. However, the difference in MIL susceptibility between pre- and post-treatment isolates was hardly 2-fold (with a maximum observed $IC_{50}$ of 12.11 μM in the post-treatment group). This is considerably lower than the observed difference in susceptibility between MIL sensitive and *in vitro* induced MIL-resistant (MIL-R) strains, in which the $IC_{50}$ can reach up to 60 μM.[12] Therefore, we consider these post-treatment and treatment failure isolates as parasites with an increased tolerance to MIL, but not (yet) as true MIL resistant. It is interesting to note that 20 (77%) of 26 patients, from whom post treatment cultures could be grown, went on to achieve initial and later final cure. Despite being infected with parasites that had a relatively higher MIL tolerance, relapse of the disease did not occur in most of these patients. For 6 patients (highlighted in bold in Table 1), paired isolates were available at both time points (pre- and post-treatment). In five of them, $IC_{50}$ values observed at the onset of treatment and the end of it (patients BHU807, 815, 869, and 994) or the relapsing time after 7 months (patient BHU828), were very similar or slightly increased. In the last patient (BHU1080), a decrease was observed at the time of treatment failure. These results thus suggests that an increased tolerance of the *L. donovani* to MIL can indeed be acquired during treatment, but that this is likely not enough to contribute to MIL relapse in the patient. It is tempting to compare these results with those of Bhandari and others,[15] who also found an increase in MIL tolerance (measured at the *in vitro* amastigote level) between pre- and post-treatment isolates of PKDL patients who are treated for 60 days instead of the 28 days for VL patients.

One might consider the strains with a higher MIL tolerance to be a stepping stone toward full blown MIL-resistance. But why do MIL-R strains still not occur after several consecutive years of MIL being the first line VL-treatment in the Indian subcontinent? Because pre-treatment isolates showed a significantly lower tolerance to MIL, these post-treatment parasites that better tolerate MIL (isolated from both cured and failed patients) apparently do not succeed in being adequately transmitted between different hosts. Such a transmission defect might be an explanation why full blown MIL-resistant strains have as yet not been described in the Indian subcontinent because transmission may favor different rounds of MIL-treatment in different host and subsequently the acquisition of a gradually higher MIL-tolerance. Losing their more MIL-tolerant phenotype might also be a valid hypothesis if the MIL-tolerant phenotype is caused by the presence of specific episomes for example, which have shown to be able to decrease significantly when drug pressure is reduced.[16] However, the question on which factors contribute to MIL-failure still remains because parasite susceptibility to MIL could not be correlated to MIL-treatment outcome of the patient—studies on MIL-blood levels[9] and other traits of the infecting parasite might thus throw a better light on the underlying factors that contribute to MIL-fail of VL patients.

In conclusion, we applied a validated and standardized *in vitro* promastigote MIL-susceptibility test to study the relation between MIL-treatment outcome of the patient and parasite susceptibility to MIL. We could not correlate MIL relapse to the presence of MIL-R strains in the affected patients, but did observe that parasites may acquire a slight increase in parasite tolerance to MIL during MIL treatment of the patient. These parasites with a higher MIL-tolerance, which cannot be considered to be MIL-R, may contribute to the future emergence of true MIL-R strains but future studies on their transmissibility are required. Nonetheless, our data prove that more tolerant MIL parasites can indeed emerge, warranting the need for continuous close epidemiological monitoring of *L. donovani* MIL susceptibility in the Indian subcontinent.

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